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# Expression of human peripheral cannabinoid receptor for structural studies

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## Abstract

Human peripheral-type cannabinoid receptor (CB2) was expressed in *Escherichia coli* as a fusion with the maltose-binding protein, thioredoxin, and a deca-histidine tag. Functional activity and structural integrity of the receptor in bacterial protoplast membranes was confirmed by extensive binding studies with a variety of natural and synthetic cannabinoid ligands. *E. coli* membranes expressing CB2 also activated cognate G-proteins in an in vitro coupled assay. Detergent-solubilized receptor was purified to 80%–90% homogeneity by affinity chromatography followed by ion-exchange chromatography. By high-resolution NMR on the receptor in DPC micelles, it was determined that purified CB2 forms 1:1 complexes with the ligands CP-55,940 and anandamide. The receptor was successfully reconstituted into phosphatidylcholine bilayers and the membranes were deposited into a porous substrate as tubular lipid bilayers for structural studies by NMR and scattering techniques.

**Keywords:** cannabinoid receptor; CB2; *E. coli*; NMR; GPCR; anodic aluminum oxide

The peripheral cannabinoid receptor, CB2, belongs to the class of G-protein-coupled receptors (GPCRs). These are integral membrane proteins involved in a large number of physiological processes including sensory transduction, cell-to-cell communication, and immune and hormonal response (Martin 1986; Munro et al. 1993). GPCRs have become the object of intense investigations, in part because of their role as therapeutically important targets of drugs. One of the major

difficulties in rational design and synthesis of potential modulators of GPCR activity is the lack of information on GPCR structure. It is notoriously difficult to crystallize GPCRs, most likely because their structure and conformation are intricately linked to the surrounding lipid matrix. Furthermore, in GPCR crystals the loop regions that connect the seven transmembrane helices are structurally heterogeneous (Ohguro et al. 1996), which prevents obtaining information about this important site by crystallography. Another limitation is that GPCRs in crystals are not functional.

Here we explored the feasibility of expressing, purifying, and reconstituting CB2 for structural studies at functional conditions by methods like nuclear magnetic resonance and scattering techniques. With the notable exception of rhodopsin, most of the GPCRs are not available in large quantities from natural sources. Therefore, their expression in a heterologous host constitutes a more practical alternative. *Escherichia coli* offers important advantages as a potential expression host, including robustness of cultivation, ease of constructing expression strains, and the absence of expensive media requirements.

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**Abbreviations:** CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHS, cholesteryl hemisuccinate Tris salt; GPCR, G-protein-coupled receptor; H10, deca-histidine tag; DM, *n*-dodecyl- $\beta$ -D-maltoside; MBP, *E. coli* maltose-binding protein; OG, *n*-octyl- $\beta$ -D-glucopyranoside; Tev, tobacco etch virus; TrxA, *E. coli* thioredoxin; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPC, dodecylphosphocholine; MOPS, 3-(*N*-Morpholino)propanesulfonic acid.

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Besides the availability of a variety of auxotroph strains, *E. coli* provides an option for selective incorporation of labeled amino acid residues into the heterologously expressed protein(s), which makes this approach especially attractive for subsequent investigations by NMR and neutron scattering (Broos et al. 1999, 2003; Rajesh et al. 2003).

Attempts at heterologous expression of the central, CB1, as well as of the peripheral, CB2, cannabinoid receptor in *E. coli* have been reported (Calandra et al. 1997). Although CB2 exhibits high levels of similarity to CB1 within the transmembrane regions (68% identity), the overall identity through the polypeptide sequences of both receptors is only 44%. It appears that differences in primary structure between the central and peripheral receptors result in significant differences in their expression levels. Expression of CB2 as a fusion with maltose-binding protein yielded significant levels of functional receptor as judged by a binding study using one ligand (Calandra et al. 1997). Expression levels of CB1 fused to MBP were at least an order of magnitude lower, and the expressed recombinant receptor appeared to lack functionality (Calandra et al. 1997). The expression of CB2 in yeast *Pichia pastoris* was reported recently, but attempts to purify the recombinant protein did not yield a functional receptor (Feng et al. 2002).

Here we describe a robust and reliable methodology for expression and purification of functionally active CB2 with yields in the milligram range as well as the preliminary results of successful reconstitution of CB2 into porous substrates for structural studies in functional conditions.

## Results

### *E. coli* expression of CB2 fusion protein with N- and C-terminal tags

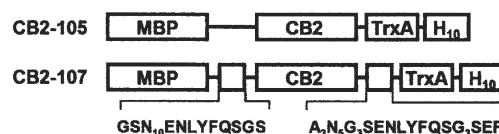
A successful approach to functional expression of GPCRs in *E. coli* was described previously (Calandra et al. 1997). It included the use of the N-terminal fusion of the GPCR with maltose-binding protein (MBP) of *E. coli* that apparently facilitates correct folding and insertion of the recombinant fusion protein into the cytoplasmic membrane. A C-terminal hexa-histidine tag was included for subsequent purification by immobilized metal affinity chromatography. In subsequent studies, this combination of tags was expanded by addition of the TrxA sequence at the C-terminal part of the GPCR, which resulted in higher stability of the fusion protein. The C-terminal hexa-histidine tag was later substituted by the deca-histidine tag, which reportedly resulted in a tight binding of the recombinant protein on a Ni-NTA agarose column and raised the purity of the recombinant recep-

tors significantly (Grishammer et al. 1999; White et al. 2004).

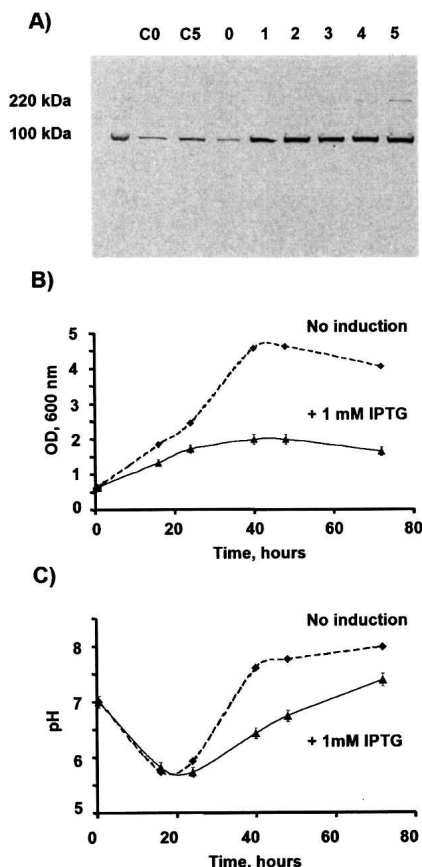
Incorporation of the two tobacco etch virus (Tev) protease recognition sites into the sequence of recombinant polypeptide upstream and downstream of the GPCR sequence, respectively, provided a means to remove the N- and C-terminal tags by the *in vitro* action of a specific protease (White et al. 2004). Cleaving of the fusion has advantages since the presence of large tags (such as MBP and TrxA) in the purified fusion protein can negatively affect the efficiency of sample preparation of GPCRs for subsequent structural studies (reconstitution into the lipid matrix).

We have constructed two plasmids for expression of the human peripheral cannabinoid receptor CB2, namely, pAY105 and pAY107 (see Materials and Methods; Fig. 1). Both plasmids are based on a pMAL-p2 vector backbone (low-copy number plasmid). They encode the MBP fused to the N-terminal part of CB2, and TrxA followed by the deca-histidine tag, fused to the C-terminal end of CB2. Expression of the fusion protein is controlled by the *lac* promoter and is induced by the addition of IPTG. The plasmid pAY107 encodes additionally two Tev recognition sites located upstream and downstream of the CB2 sequence, respectively. Cleavage of the CB2-107 fusion protein at these sites should produce CB2 polypeptide with addition of Ser-Gly-Ser at the N-terminal end and Ala<sub>3</sub>-Asn<sub>5</sub>-Gly<sub>3</sub>-Ser-Glu-Asn-Leu-Tyr-Phe-Gln at the C-terminal end.

Expression of the CB2-105 and CB2-107 was attempted in *E. coli* strains DH5 $\alpha$  and a protease-deficient strain BL21 (DE3). Expression of the CB2 fusion protein was induced by addition of 1 mM IPTG, and incubation was carried out at 20°C in order to facilitate correct folding and membrane insertion of the recombinant receptor. Accumulation of the fusion protein in cytoplasmic membranes was monitored by Western blot using antibodies against MBP and the N-terminal part of CB2. Additionally, the recombinant protein was detected by action of a specific reagent, INDIA-His-Probe-HRP, reacting with the poly-histidine tag and visualized using DAB-peroxide (Pierce). Levels of the recombinant CB2 in *E. coli* (normalized per total protein) increased steadily during induction, up to 24 h. At longer times no further increase in accumulation of a full-length recombinant protein was observed (Fig. 2).



**Figure 1.** Schematic representation of the CB2 receptor fusion proteins.



**Figure 2.** Time course of CB2-107 expression in *E. coli*. (A) An overnight culture of BL21-107 cells was used to inoculate several incubation flasks containing equal volumes of media. Induction was started when cell density reached OD<sub>600</sub> = 0.4. Cell samples were collected at various time points before and after the induction with IPTG. Samples containing an equal number of cells were lysed in Laemmly sample buffer and proteins separated on a 10% SDS-PAGE. After electroblotting onto the nitrocellulose membranes, proteins were probed with anti-His INDIA-HRP reagent. C<sub>0</sub> and C<sub>5</sub>, control cells without addition of IPTG at time points 0 and 72 h. (Lanes 1–5) Induced cells at time points: 0, 16, 24, 40, 48, and 72 h, respectively. (B) Time course of BL21-107 cell growth as measured by changes in OD<sub>600</sub>. (Dotted line) Cells without induction; (solid line) cells induced with 1 mM IPTG. (C) pH of the incubation media during the incubation of BL21-107.

However, accumulation of cellular biomass continued after 24 h, reaching a maximum ~40 h after induction. More prolonged incubation (up to 72 h) did not result in further accumulation of biomass nor did it increase the levels of recombinant receptor. Therefore, in order to maximize the yield of the recombinant receptor, in subsequent experiments cells were collected at 40 h after the beginning of induction. Preliminary experiments performed on CB2-105 and CB2-107 revealed no significant differences between these two expression constructs in either levels of accumulated recombinant receptor or in the density of the ligand-binding sites on *E. coli*

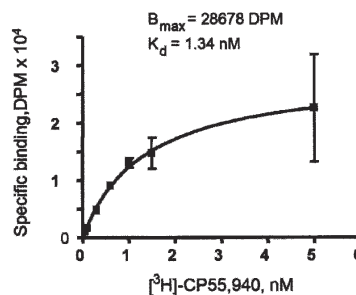
membranes (data not shown). Therefore, all subsequent studies were performed with expression plasmid CB2-107.

Use of the protease-deficient strain BL21-107 resulted in some twofold higher accumulation of biomass after 40 h of induction when compared to the DH5 $\alpha$ /pAY107 (data not shown). Because relative cellular levels of the recombinant CB2-107 receptor accumulated in these two strains were almost identical, strain BL21-107 was chosen for subsequent expression studies.

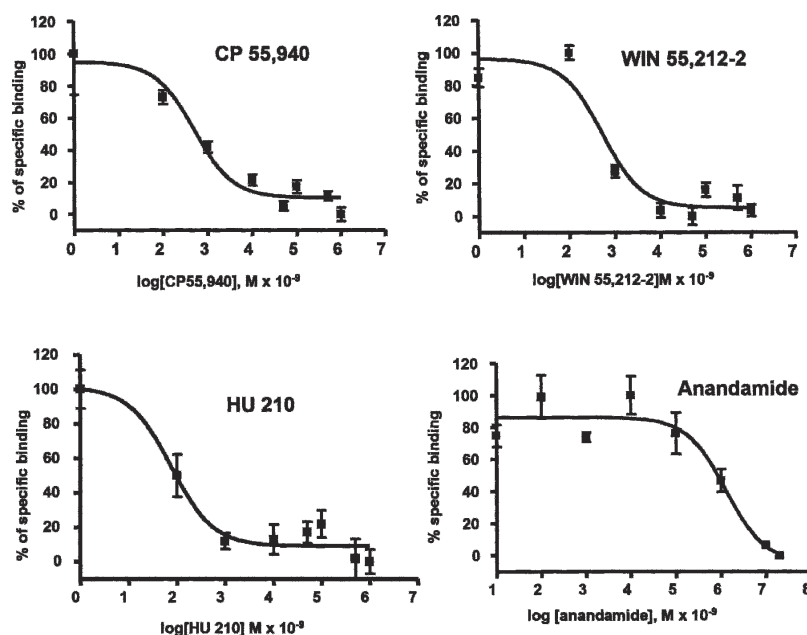
#### Ligand-binding assay on *E. coli* BL21-107 membranes

In order to determine the functional activity and structural integrity of the recombinant CB2 receptor expressed in *E. coli*, ligand-binding studies on a large number of ligands were performed. Ligand-binding assays on the *E. coli* BL21-107 membranes were carried out as described in Materials and Methods. [<sup>3</sup>H] CP-55,940, a specific synthetic ligand for the peripheral cannabinoid receptor, was used in both saturation and competitive binding experiments.

The density of the ligand-binding sites in membrane preparations was calculated from the saturation binding with BL21-107 (Fig. 3) and varied from 5 to 13 pmol per mg of membrane protein depending on the preparation, corresponding to a content of up to 0.2% of recombinant CB2 in the fraction of membrane proteins, assuming one binding site per molecule of recombinant receptor. Recombinant CB2 receptor displayed high affinity binding of [<sup>3</sup>H] CP-55,940 with a  $K_d$  of 1.3 nM. This result correlates well with the reported  $K_d$  values (0.7–3.7 nM) for the native CB2 receptor preparations from various tissues (Felder et al. 1995). The membranes of the untransformed BL21 (DE3) cells did not exhibit any specific binding of [<sup>3</sup>H] CP-55,940 (data not shown). The nonspecific binding was determined in the presence of 1  $\mu$ M of unlabeled CP-55,940 and constituted about 40%–50% of total binding.



**Figure 3.** Saturation binding of [<sup>3</sup>H] CP-55,940 on BL21-107 membranes expressing CB2. The assay was performed in triplicate as described in Materials and Methods.



**Figure 4.** Competitive binding of cannabinoid ligands on BL21-107 membranes expressing CB2. The assay was performed in triplicate as described in Materials and Methods.

We have further studied displacement of [<sup>3</sup>H] CP-55,940 from BL21-107 membranes with various specific and nonspecific ligands of CB2 (Fig. 4; Table 1). Nonselective synthetic agonists HU210 and WIN 55,212-2 exhibited high affinity binding with  $K_i$  values of 0.07 and 0.5 nM, respectively. The  $K_i$  value for JWH 015, a selective synthetic CB2 agonist, was 10.4 nM, in good agreement with the reported value of 13.8 nM (Chin et al. 1999). On the other hand, the key synthetic CB1 agonist ACEA did bind to the recombinant CB2 with a  $K_i$  of 164 nM, a significantly higher affinity than the literature value for  $K_i$  of  $> 2 \mu\text{M}$  (Pertwee 2001). Recombinant CB2 exhibited low affinity for endogenous cannabinoid agonists anandamide and noladin ether, with the  $K_i$  values of 7.6  $\mu\text{M}$  and 4.3  $\mu\text{M}$ , similar to the values reported for the native cannabinoid receptor (Pertwee 2001).

Recombinant CB2 binds cannabinoid agonist (-)- $\Delta^9$ -tetrahydrocannabinol with moderately high affinity ( $K_i = 39.8 \text{ nM}$ ). Two other cannabinoid ligands, cannabidiol and abnormal (Abn)-cannabidiol, exhibit low affinity binding, with  $K_i$  values exceeding 2  $\mu\text{M}$ .

AM630, a key CB2 antagonist, exhibited moderate binding with a  $K_i$  of 216 nM. Selective CB1 antagonist AM281 exhibited low affinity binding with a  $K_i$  of 2.7  $\mu\text{M}$ . Another CB1 antagonist, AM251, exhibited moderate affinity with a  $K_i$  of 146 nM, unlike the value of  $> 2 \mu\text{M}$  reported in literature for native CB2 receptor (Lan et al. 1999). All ligand-binding experiments were performed in triplicates, and the variability of the results was within 10% of the reported values.

#### *Reduction of nonspecific ligand binding by formation of single, tubular lipid bilayers*

The conventional competitive filter-binding assay resulted in significant nonspecific binding (up to 50%) of the hydrophobic ligand (CP-55,940) on the filter that increased the experimental error limits of the ligand-binding parameters  $K_d$  and  $B_{\text{max}}$ . Recently we demonstrated formation of single, tubular lipid bilayers in aluminum oxide-based Anopore filters with a pore size of 0.2  $\mu\text{m}$  (Whatman) (Gaede et al. 2004; O. Soubias, I.V. Polozov, W.E. Teague, and K. Gawrisch, unpubl.). Here we ex-

**Table 1.** Ligand-binding studies on membranes of *E. coli* cells expressing CB2-107

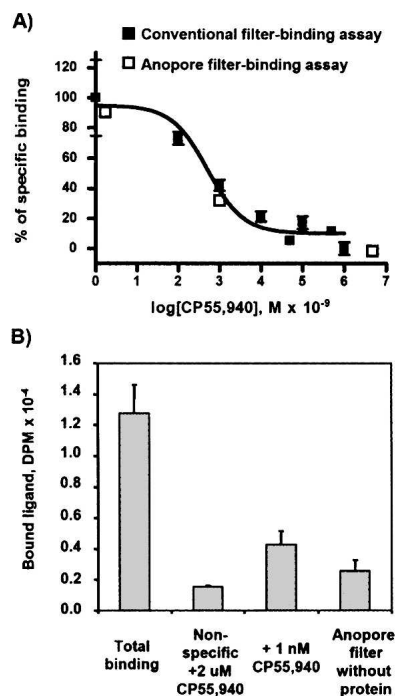
Competing ligand	$K_i$ (nM)	EC50 (nM)	$K_i$ reported in literature (nM)	Reference
CP 55,940	0.2–0.63	0.25–0.71	0.78–3.96	Pertwee 2000
HU 210	0.070	0.091	0.084–0.098	Pertwee 1999
WIN 55,212-2	0.51	0.66	0.3–4.1	Pertwee 2001
SR 141716A	3430	4710	$> 1 \mu\text{M}$	Pertwee 1999
JWH 015	10.4	12.9	13.8	Chin et al. 1999
$\Delta^9$ -THC	39.8	51.8	26–76	Pertwee 2001
AM251	146	198	2290	Lan et al. 1999
AM630	218	288	32	Pertwee 2001
ACEA	164	215	$> 2000$	Pertwee 2001
2-AG ether	4330	5950	$> 3000$	Pertwee 2001
Anandamide	7620	9483	2597–6260	Pertwee 2001
AM281	2755	3551	4200	Pertwee 2001
Cannabidiol	2188	2882	No data	
Abn-cannabidiol	6680	8308	No data	

plored if nonspecific ligand binding is reduced when experiments are conducted on single, tubular lipid bilayers that line the inner surface of pores.

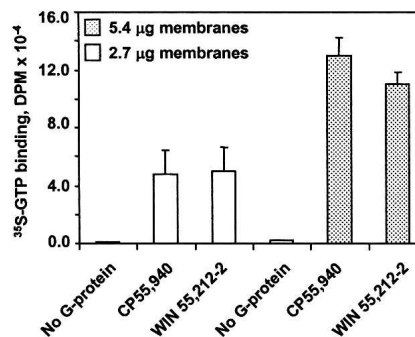
Samples were prepared by extrusion of bacterial protoplast membranes through Anopore filters. By  $^1\text{H}$  MAS NMR we confirmed that filters had been loaded with membranes. Loading of the membranes with radioactive CP-55,940, removal of unbound ligand, and scintillation analysis of radioactivity in the Anopore filters were conducted similar to the conventional assay. The ligand-binding parameters of CB2 in Anopore filters were identical to the parameters of the conventional filter assay (Fig. 5A). This indicates that deposition of membranes on the Anopore surface did not interfere with CB2 function. As expected, nonspecific binding measured on single tubular bilayers in Anopore was much lower. Total binding exceeded nonspecific binding, measured in the presence of large excess of unlabeled ligand, by a factor of 8 (Fig. 5B). Thus, tubular bilayers in Anopore offer significant promise as solid support in receptor–ligand interaction studies.

#### Activation of G-proteins in an *in vitro* coupled assay

In order to assess the functionality of the recombinant CB2 in *E. coli* membranes, we reconstituted the mem-



**Figure 5.** Ligand-binding assay on Anopore filters. (A) Comparison of the conventional filter-binding assay performed on Whatman GF/B filters (■) and ligand binding performed on Anopore membranes (□). (B) Competition binding of [ $^3\text{H}$ ] CP-55,940 on Anopore filters loaded with BL21-107 membranes expressing CB2.



**Figure 6.** Activation of G-proteins by the *E. coli* membranes expressing CB2. The assay was performed as described in Materials and Methods using either 2.7 or 5.4 μg of urea-treated membranes per sample. Background (binding of [ $^3\text{S}$ ]GTPγS to  $G_{\alpha i}$  in the absence of CB2) was subtracted. All measurements were performed in triplicate.

brane preparation of the receptor *in vitro* with heterotrimeric G-proteins. The receptor was activated by the addition of specific cannabinoid agonists CP-55,940 or WIN 55,212-2, and activation of the G-proteins was studied by measuring the activity of [ $^3\text{S}$ ]GTPγS captured by the receptor–G-protein complex (Fig. 6). Urea-treated membranes were used in these experiments to reduce the nonspecific activation of G-proteins (Glass and Northup 1999). Purified G-protein subunits ( $G_{\alpha i}$  and  $G_{\beta 1}$   $G_{\gamma 1}$ ) were kindly provided by Dr. J. Northup (NIDCD, NIH). Specific activation of G-proteins by the recombinant CB2 was observed in the presence of either of the cannabinoid agonists CP-55,940 or WIN 55,212-2. The activation of  $G_{\alpha i}$  increased proportionally to receptor concentration (Fig. 6) as judged from the binding of [ $^3\text{S}$ ]GTPγS, thus indicating that the assay was limited by the amount of receptor. No agonist-stimulated binding of the [ $^3\text{S}$ ]GTPγS was observed in the absence of expressed CB2 (data not shown). These results provide solid evidence for functionality and structural integrity of the recombinant fusion CB2 receptor in *E. coli* membranes.

#### Solubilization and purification of CB2-107 fusion protein

Solubilization and purification of the CB2-107 fusion protein was performed from BL21-107 cells as described in Materials and Methods. Solubilization of CB2-107 was achieved by using a mixture of three detergents: CHAPS, DM, and CHS. It was demonstrated that the presence of 0.1% CHS during solubilization and purification is an important factor in preserving the functional activity and structural integrity of recombinant GPCRs (Grisshammer and Tucker 1997; Grisshammer et al. 1999; Weiss and Grisshammer 2002). All buffers

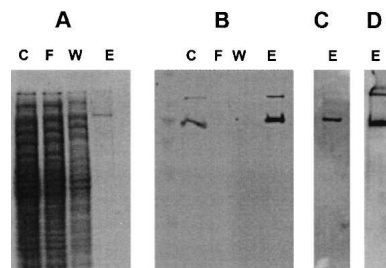
used for solubilization and purification also contained 30% (v/v) glycerol, which is generally believed to have a stabilizing effect on solubilized membrane proteins.

Frozen BL21-107 cells expressing recombinant CB2 receptor were resuspended in the solubilization buffer and subjected to mild sonication. Solubilized proteins were separated from insoluble particles/cell debris by ultracentrifugation. Efficient solubilization and recovery of up to 80%–90% of the recombinant receptor, as determined by the Western blot analysis, was achieved. Direct solubilization of CB2-107 from the intact cells rather than from the cytoplasmic membranes was employed in order to bypass the time-consuming procedure of preparation of a cell membrane fraction, and to preserve a large portion of the receptor in a functional form.

Immobilized metal affinity chromatography on a Ni-NTA agarose column (QIAGEN) was used as the first step of the purification procedure. The presence of the deca-histidine tag at the C-terminal end of the fusion protein allowed for tight binding of CB2-107 to the resin. Binding was performed from the solubilization buffer (see Materials and Methods), which contained 200 mM NaCl. No imidazole was included in the buffer during the binding step since the presence of even low concentrations (30 mM) of imidazole resulted in a noticeable decrease in the amount of recombinant CB2-107 retained on a Ni-NTA agarose column (data not shown). Resin retaining fusion protein was extensively washed with 30 mM imidazole in solubilization buffer, and recombinant receptor was eluted with a linear gradient of imidazole. Most of the fusion protein was eluted with imidazole concentrations between 80 and 150 mM.

The purity of the recombinant protein was assessed by SDS-PAGE and by Western blot probed with antibodies against MBP, CB2, and INDIA-HRP reagent. Small-scale purifications typically yielding 1–2 mg of recombinant receptor resulted in an 80%–90% homogeneous preparation of CB2, as judged by SDS-PAGE (Fig. 7). Scaling up of the purification protocol, in order to produce 10 mg of fusion protein or more, resulted in a decrease in the overall purity of the final preparation, yielding a 50%–60% homogeneous preparation of CB2-107.

We have attempted to further purify the recombinant CB2 by subjecting it to a second chromatography step. The CB2-107 fractions eluted from the Ni-NTA agarose were combined and concentrated ~10-fold on a centrifugal concentrating device, Apollo, 20 mL, 70 kDa molecular weight cutoff (Orbital Biosciences). This protein-concentrating procedure resulted also in an almost proportional eightfold to 10-fold increase in the concentration of detergents as determined by NMR studies on the micellar solution. Combined fractions were then diluted 10-fold with the Tris-HCl (pH 7.5) buffer containing 30% glycerol, in order to bring the concentration



**Figure 7.** Purification of fusion CB2 on Ni-NTA agarose: SDS-PAGE and Western blot analysis. (A) SDS-PAGE of fractions from Ni-NTA agarose: (lane C) crude extract applied onto Ni-NTA column; (lane F) flowthrough fractions; (lane W) wash fractions; (lane E) elution with 200 mM imidazole. (B) Western-blot analysis of fractions from the Ni-NTA column. The membrane was probed with anti-MBP antibody. Lanes C, F, W, and E are the same as in A. (C,D) Western-blot analysis of protein eluted from Ni-NTA column probed with anti-CB2 antibodies (C) or INDIA-HRP anti-His reagent (D).

of NaCl in the sample to below 20 mM, and applied onto the ion-exchange resin. Chromatography was performed on an anion-exchange resin Source 30Q. Recombinant protein was retained on a column at 20 mM NaCl, and eluted with a linear ascending gradient of NaCl in the binding buffer. Recombinant CB2 was eluted as a broad peak at the beginning of the gradient at concentrations of NaCl between 35 and 60 mM. Typically, this second purification step resulted in only slight improvement in the overall purity of the recombinant CB2 to 85%–90%. Our subsequent attempts to increase the efficiency of the second chromatography step by using different types of anion-exchange columns as well as hydrophobic chromatography did not result in a noticeable improvement of purity. Some minor losses of protein (~10%) were also observed as a result of this purification procedure.

#### *Internal peptide sequencing of the purified CB2-107*

Confirmation of the structure of the recombinant CB2 expressed in *E. coli* was obtained by internal peptide sequencing of the purified fusion CB2, digested with trypsin. A proteolytic digest of the CB2, liquid chromatographic separation, and tandem mass spectrometry analysis of the resulting peptides, as well as the database search were performed by ProTech, Inc. A total of 20 peptides was identified, 15 of them matching the sequence of the periplasmic maltose-binding protein, three matching the human peripheral cannabinoid receptor, and two matching thioredoxin, thus providing an unambiguous confirmation for the primary amino acid sequence of the expressed and purified recombinant receptor CB2 (Table 2).

**Table 2.** CB2-107 internal peptide sequenced by nano LC-MS/MS

Peptide sequence	Matching amino acids in CB2-107 sequence	Matching sequence in database
VTVEHPDKLEEK	61–72	gi[3983122], maltose-binding protein (E. coli)
YDIKDVGVNAGAK	202–215	
HMNADTDYSIAEAFNK	229–245	
TWEEIPALDK	155–162	
GQPSKPFVGVLSAGINAASPNK	278–299	
VNYGVTVLPTFK	266–277	
FGGYAQSGLLAEITPDK	93–109	
LYPFTWDAVR	115–124	
FGGYAQSGLLAEITPDKAFQDK	93–114	
AFQDKLYPFTWDAVR	110–124	
FPQVAATGDGPDIIFWAHDR	73–92	
EFLENYLLTDEGLEAVNK	304–321	
ELAKEFLYLLTDEGLEAVNK	300–321	
AGLTLFLVDLIK	216–228	
LIAYPIAVEALSLIYNK	129–145	
GLGSEAKEEAPR	735–746	gi[46854759], cannabinoid receptor 2 (Homo sapiens)
SSVTETeadgKITPWPDSR	747–766	
DYMILSGPQK	436–445	
LNIDQNPGTAPK	855–866	gi[230778], thioredoxin (E. coli)
IIHLTDDSFDTDLVK	801–815	

#### N-terminal sequencing of the purified CB2-107

N-terminal sequencing by Edman degradation of the purified CB2-107 after electroblotting it onto the PVDF membrane (Immobilone-P, Millipore) was performed by Midwest Analytical, Inc. The obtained sequence, KIEEGKLIWIN, matched the sequence of the mature maltose-binding protein of *E. coli* (after removal of the signal peptide).

#### Removal of N- and C-terminal tags and preparation of CB2 protein

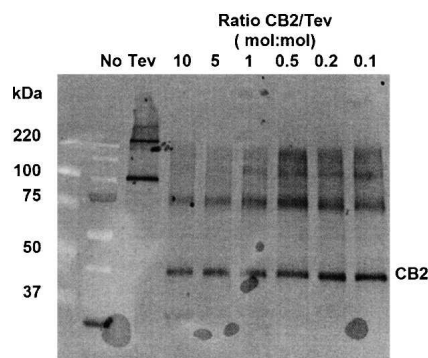
To obtain a preparation of CB2 receptor devoid of the N-terminal MBP and C-terminal TrxA-deca-histidine tags, the purified fusion CB2-107 was treated with specific Tev protease. Proteolytic cleavage of the fusion protein was monitored by SDS-PAGE and Western blot analysis of reaction products (Fig. 8). Preliminary studies using commercially available preparations of Tev protease revealed insufficient activity of this enzyme in the presence of high concentrations of detergents (CHAPS, CHS, DM) at the reaction temperature of 4°C. In order to overcome this problem, we expressed

and purified a mutated form of Tev protease (S219V) that is characterized by high stability, and is not prone to self-proteolysis. Efficient cleavage of the fusion CB2 protein occurring at both proteolytic sites was detected after incubation with Tev protease at 4°C for several hours. A major proteolytic product with the molecular mass of 40 kDa was detected by SDS-PAGE and Western-blot-probed with antibodies against CB2. The presence of high concentrations of detergents, glycerol, and 100 mM NaCl did not have a significant detrimental effect on the efficiency of the reaction. A minor proteolytic product with the mobility in SDS-PAGE corresponding to a polypeptide of ~80 kDa was detected in all samples, indicating that the most efficient cleavage occurred at the second Tev site separating the C-terminus of CB2 and TrxA.

#### Ligand-binding studies in the micellar state by high-resolution NMR

Functionality of the purified fusion CB2 in the micellar state was determined by studying its interactions with cannabinoid ligands by solution-state NMR. Care was taken to record NMR spectra with the utmost level of reproducibility. First a spectrum of CB2 in micelles of deuterated DPC without added ligand was acquired at a high level of signal to noise. Then spectra as a function of ligand concentration were recorded. Detergent concentration in the ligand stock solution was matched to the detergent concentration of the CB2 solution to prevent spectral changes. The spectrum of CB2 without added ligand was subtracted from the ligand containing spectra yielding the resonances of the ligand as well as of DMSO, which was added to the ligand stock solution as an internal concentration standard. We had established earlier that DMSO does not interact strongly with CB2.

Experiments were conducted in the concentration range from 13 to 37  $\mu$ M for CB2 and 1–100  $\mu$ M for the ligands CP-55,940 and anandamide. Binding of CP-55,940 to CB2 was determined by recording the integral



**Figure 8.** Cleavage of the CB2 fusion protein with Tev protease. The Western blot was probed with antibodies against CB2.

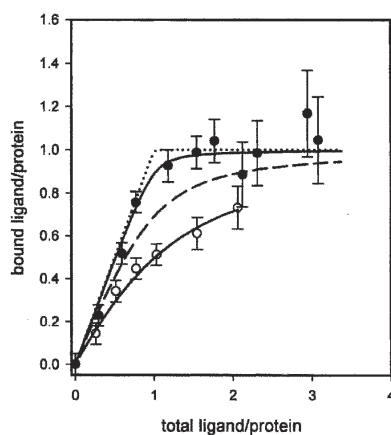
intensity of the aromatic resonance at 7.03 ppm. At ligand concentrations less than the concentration of active CB2, the signal at 7.03 ppm could not be detected because the ligand resonance was broadened beyond detection. As soon as the ligand concentration exceeded the concentration of active CB2, a narrow resonance of free (unbound) ligand appeared. The difference between total and free ligand concentrations yielded the concentration of binding-competent CB2. The experiment was repeated three times. Although the concentration of CB2 varied between samples by about a factor of 3, the data were perfectly superimposable after dividing ligand concentration by the concentration of ligand-binding-competent CB2. The graph combining the results of all three experiments is shown in Figure 9.

Experiments on binding of the endogenous ligand anandamide to CB2 were conducted on protein samples from the same stock solutions as for CP-55,940. The anandamide resonance of the methylene protons between the double bonds (resonance frequency 2.8 ppm) was recorded. Results were similar to CP-55,940 binding except for a less-well defined breakpoint at the concentration of active CB2 (see Fig. 9).

The Law of Mass Action, assuming formation of a 1:1 complex between ligand and CB2, yields the following equation:

$$L_b = (L_t + P + K_d)/2 - \sqrt{(L_t + P + K_d)^2/4 - L_t \cdot P},$$

where  $L_b$  is the concentration of bound ligand;  $L_t$  and  $P$  are the concentrations of total ligand and ligand-binding



**Figure 9.** Ligand binding to purified, recombinant CB2 in the micellar state. On the X-axis the concentration of ligand divided by the concentration of ligand-binding-competent CB2 is reported. On the Y-axis the concentration of ligand bound to CB2 is shown (●, CP-55,940; ○, anandamide). The solid lines are a fit to the data points assuming formation of a 1:1 complex between CB2 and ligands (CP-55,940,  $K_d = 5 \times 10^{-7}$  M; for comparison, the curve for  $K_d = 5 \times 10^{-6}$  M [dashed line] and  $K_d = 5 \times 10^{-10}$  M [dotted line] are shown as well; anandamide,  $K_d = 1 \times 10^{-5}$ ).

sites on the protein, respectively; and  $K_d$  is the ligand dissociation constant. The solid lines in Figure 9 are a fit of the experimental data to the equation above. The well-defined breakpoint in the CP-55,940 binding curve at the concentration of binding competent CB2 results from high ligand affinity in combination with high protein concentrations in the micromolar range. The concentration of binding-competent CB2 was in the range from 13  $\mu$ M to 37  $\mu$ M for the three investigated samples, corresponding to at least 85% of purified CB2 protein. Total protein content was determined by a protein assay. The fit yielded a  $K_d$  of  $5 \times 10^{-7}$  M or lower for CP-55,940 and of  $1 \times 10^{-5}$  M for anandamide. For comparison, binding curves assuming  $K_d$  values of  $5 \times 10^{-6}$  M (dashed line) and  $5 \times 10^{-10}$  M (dotted line) are shown as well. While the lower  $K_d$  value quite possibly may fit the data points of CP-55,940 binding, the higher value clearly does not. There was a remarkably precise agreement on the concentration of binding-competent CB2 for all three samples between experiments on CP-55,940 and anandamide. The observations strongly suggest that CB2 forms a 1:1 complex with both CP-55,940 and anandamide in micellar solution. The dissociation constant of anandamide in micellar solution is in reasonable agreement with the values measured for recombinant CB2 in the bacterial membrane. The dissociation constant of CP-55,940 in micellar solution could be similar to values obtained for bacterial membranes as well. However, because of the higher protein concentration required for NMR experiments, we were unable to determine such low  $K_d$  values with sufficient precision.

Ligand-binding studies on the stronger binding ligand, HU210, were conducted as well. While addition of the first two increments of ligand suggested some binding as seen in a decrease of intensity of aromatic HU resonances, signal intensity fully recovered after addition of equimolar, relative to active protein, amounts of HU210. Furthermore, a weak aromatic resonance was detected that could not be assigned to either resonances of HU210 or to the spectrum of CB2. The apparent lack of ligand binding as well as the appearance of this additional resonance suggest that long-term chemical stability (24 h) of either ligand or protein are compromised in the presence of HU210. This prevented us from conducting meaningful binding experiments on this ligand. Attempts to competitively displace CP-55,940 by addition of HU210 were unsuccessful as well.

#### *Reconstitution of the purified recombinant CB2 into a lipid matrix*

Reconstitution of the purified, recombinant CB2 into the lipid matrix is desirable for subsequent structural

characterization of the receptor. For reconstitution of CB2 into lipid bilayers, we followed procedures for another GPCR, the visual receptor rhodopsin. Briefly, a receptor/lipid dispersion in OG micelles is rapidly diluted below the critical micelle concentration (CMC), resulting in formation of small proteoliposomes (Jackson and Litman 1985). In order to facilitate the detection of proteoliposomes formed upon dilution of the detergent–protein–lipid mixture, as well as the incorporation of the CB2 into the lipid matrix, a small fraction of both lipid and protein were labeled with fluorescent dyes as described in Materials and Methods. Proteoliposomes containing CB2 were formed by rapid dilution of the micellar solution below the CMC of OG.

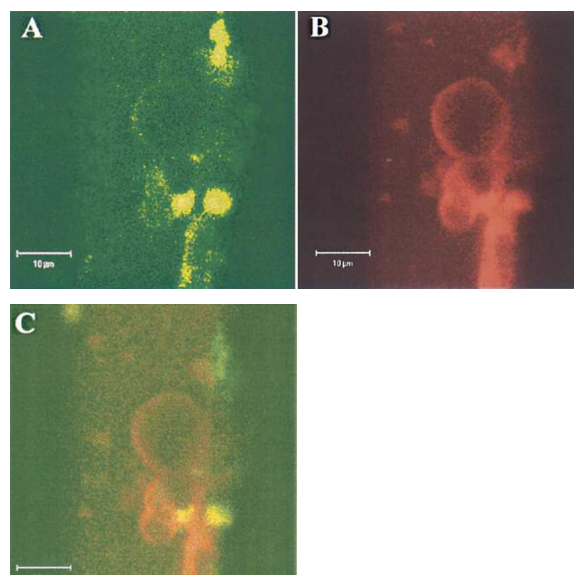
#### *Confocal microscopy of the CB2-containing liposomes*

Confirmation of the successful reconstitution of the CB2 into the lipid matrix was obtained from studying proteoliposomes containing labeled CB2 by confocal microscopy. Liposomes were obtained by mixing of the fluorescently labeled CB2 with SOPC/TexasRed-labeled 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine in OG and dilution below the CMC. The liposomes were concentrated by ultracentrifugation, dried on a glass slide, and rehydrated using a small quantity of water. A low-frequency electric current was applied to the two platinum wires traversing the slide to facilitate formation of large unilamellar liposomes that budded from the wire surface. The confocal images, with resolution for CB2 and lipid fluorescence labels, are shown in Figure 10.

The reconstituted liposomes appear to be unilamellar, and the colocalization of two fluorescent labels confirmed successful incorporation of the recombinant CB2 into the lipid bilayer. The image also contained occasional patches with a disproportionately intense Alexa Fluor 532 signal, suggesting that a fraction of labeled CB2 had aggregated. At this point we are uncertain if this is the result of drying and rehydrating the sample or if such patches existed prior to drying.

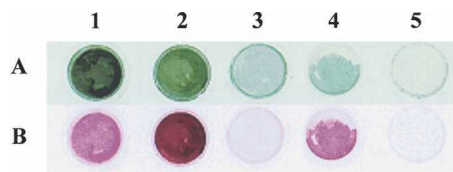
#### *Depositing of single, tubular CB2-containing bilayers into porous substrates*

Ligand-binding studies on the reconstituted CB2 as well as structural studies by NMR and scattering methods benefit greatly from immobilizing the reconstituted membranes at high concentration on a solid interface, e.g., as single tubular membranes covering the surface of filter pores (Gaede et al. 2004; O. Soubias, I.V. Polozov, W.E. Teague, and K. Gawrisch, unpubl.). Anopore filters have a very high density of uniformly distributed pores that traverse the filter disks. Their total surface area is ~500-fold larger than the disk area, which greatly



**Figure 10.** Reconstitution of fusion CB2 receptor into SOPC liposomes: confocal microscopy. Proteoliposomes were generated from Alexa Fluor 532-labeled CB2 and from Texas Red-labeled lipid. (A) Green fluorescence derived from Alexa Fluor 532; (B) red emission derived from Texas Red; (C) superimposed emission from Alexa Fluor 532 and Texas Red. Pseudocolors are used; green represents fluorescent emission of Alexa Fluor 532 and red emission of Texas Red.

benefits ligand-binding studies and raises signal intensities in structural studies. The surface properties of these filters allow efficient capture and retention of the liposomes and formation of a single lipid bilayer covering the inner surface of the pore channels. Furthermore, the large accessible surface area in the filters enables rapid removal of residual detergent from the membranes and a rapid exchange of ligand-containing solutions. After detergents were removed by extensive washing with the buffer, the amounts of lipid and protein retained on the filter were determined with a fluorescent scanner using a combination of excitation/emission filters optimal for either the lipid or protein dye. The results of a typical experiment are presented in Figure 11. Scanning of the membranes using the excitation/emission filter combination optimal for the Alexa Fluor 532-labeled protein (upper panel in the figure) confirmed deposition of proteoliposomes composed of SOPC and recombinant CB2 into Anopore filters. Control samples, containing either labeled fusion protein only (without addition of the lipid), or a mixture of the fluorescent dye with the lipid without addition of the labeled CB2, were characterized by significantly lower intensity of the fluorescent signal. Reproducibility of the reconstitution/retention in the Anopore filters was very good. The amounts of the deposited membrane protein and lipid were quantified by loading the filters with increasing quantities of the



**Figure 11.** Reconstitution of CB2 into lipid bilayer and deposition into Anopore membranes. (A) Membrane scanned at 532 nm ( $\lambda_{exc}$ ) and 580 nm ( $\lambda_{em}$ ), optimal for Alexa Fluor 532. (B) Membranes scanned at 583 nm ( $\lambda_{exc}$ ) and 610 nm ( $\lambda_{em}$ ), favoring detection of Texas Red. (1) Labeled CB2 + unlabeled lipids; (2) labeled CB2 + labeled lipids; (3) labeled CB2 (without lipid); (4) labeled lipids; (5) Alexa Fluor 532 dye. Pseudocolors are used; green represents fluorescent emission of Alexa Fluor 532 and red emission of Texas Red.

liposome dispersion. The fluorescence intensity for lipid and protein plotted versus total lipid and protein sent through the filters leveled off at 250  $\mu$ g of SOPC and 10–20  $\mu$ g of recombinant CB2 in SOPC liposomes per filter (25 mm inner diameter, 0.2  $\mu$ m pore size). This is somewhat less than the amount of lipid that could be deposited into those filters by extrusion of large multilamellar liposomes (Gaede et al. 2004). Control experiments demonstrated that the Anopore filters were able to capture and retain only trace amounts of the labeled protein, when the latter was applied in detergent micelles, thus suggesting that the procedure used to obtain liposomes by rapidly diluting the protein–detergent–lipid mixture has, indeed, allowed for significant incorporation of the labeled CB2 into a lipid matrix.

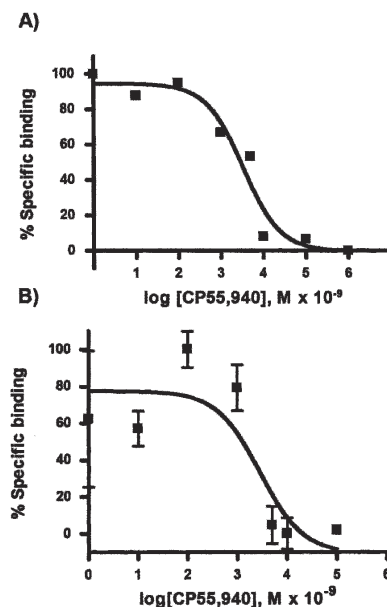
#### *Ligand-binding studies with purified, recombinant CB2 reconstituted into a lipid matrix*

The binding of agonist [ $^3$ H] CP-55,940 to CB2 reconstituted into a POPC matrix was studied. Proteoliposomes containing CB2 were obtained by dilution of the lipid–protein mixture into the cold assay buffer. Proteoliposomes were either trapped in the conventional filter-binding assay as described in Materials and Methods (lipid:CB2 ratio  $\sim$ 10,000), or the proteoliposomes were deposited as tubular bilayers into 3-mm-diameter Anopore disks (lipid:CB2 ratio  $\sim$ 2500), and then exposed to  $^3$ H-labeled CP-55,940 in the presence of variable concentrations of competing (unlabeled) CP-55,940. The results are shown in Figure 12, A and B. Both the conventional filter-binding assay and the Anopore-based assay demonstrated specific binding of the agonist to a single high affinity binding site on the reconstituted receptor. The dissociation constant was in the low nanomolar range (1.8 nM as measured in the filter-binding assay and 2.6 nM as measured in the Anopore-based assay). The count rate indicated that at least 10%–20% of the lipid-reconstituted receptor retained functionality.

Most likely the true concentration of functional CB2 is higher because the ligand-binding assay requires flushing of filters with buffer to remove unreacted ligand. Depending on ligand off-rates and the time of exposure to buffer, a certain fraction of CB2-bound ligand is lost. This is a general problem of such assays. In the future we will attempt using NMR-based ligand-binding assays that do not require removal of unbound ligand to measure the true concentration of binding-competent CB2.

#### **Discussion**

We have developed a successful procedure for production of functional recombinant human peripheral cannabinoid receptor CB2 in quantities and at purity levels sufficient to initiate structural studies. The method uses a protease-deficient strain of *E. coli* as an expression host, and requires expression of the CB2 receptor as a fusion with maltose-binding protein, thioredoxin, and a poly-histidine tag. Although such a combination of tags was previously described for the expression of another GPCR (a truncated version of the neurotensin receptor) (Grisshammer and Tucker 1997), it has not been used previously for the expression of cannabinoid receptors. We have demonstrated that the CB2 fusion protein was accumulated in *E. coli* cells at relatively high levels (1–2 mg/L), thus allowing for a large-scale expression of the recombinant protein. We have developed an efficient solubilization and purification protocol, which is suitable for



**Figure 12.** Competitive binding of CP-55,940 on purified recombinant CB2 reconstituted into a lipid matrix. (A) Conventional filter-binding assay. (B) Ligand-binding assay performed on lipid-reconstituted CB2 deposited onto Anopore membranes.

routine preparation of samples of CB2 for nuclear magnetic resonance spectroscopy as well as for diffraction methods.

The amino acid sequence of the purified CB2-fusion was verified by Western blot analysis, sequencing of internal peptides, and N-terminal sequencing. The presence of the Tev recognition sites was confirmed by proteolytic cleavage using a specific Tev-protease.

The functionality and structural stability of the expressed recombinant receptor in bacterial protoplast membranes was established in extensive ligand-binding studies. The affinity profile of the recombinant CB2 receptor determined for a large number of synthetic and natural cannabinoid ligands compares well with that reported in the literature data for CB2 present in mammalian tissues as well as for some preparations of heterologously expressed receptor (Matsuda 1997). We have found that the general order of potencies of a variety of synthetic and natural cannabinoid ligands for the recombinant receptor (HU210 > CP-55,940 >  $\Delta^9$ -THC > anandamide) was identical to that reported for the CB2 receptor found in mammalian tissues.

The affinity of the recombinant receptor for two cannabinoid ligands (AM251 and ACEA) was higher than the value reported in the literature. Synthetic cannabinoid antagonist AM251 exhibited moderate affinity for the recombinant CB2 ( $K_i$  of 146 nM), which is an order of magnitude higher than the value reported in literature (Pertwee 2001). Interestingly, two other structurally close antagonists, AM281 and SR141716A, had  $K_i$  values in the low micromolar range, similar to previously reported affinity data (Lan et al. 1999; Pertwee 2001). ACEA, a chlorine-containing analog of anandamide, binds to the recombinant CB2 with moderate affinity ( $K_i$  = 164 nM), while binding of anandamide was significantly weaker, in the low micromolar range.

Only one of the compounds tested, the synthetic antagonist AM630 had lower affinity than previously reported (Ross et al. 1999). The relatively minor discrepancies between the affinity data for the three ligands AM251, AM630, and ACEA may be attributed in part to differences in sample preparation and binding assays that are known to generate significant variability. It is also possible that the lipid environment of the *E. coli* membranes as well as the presence of the large tags influences the accessibility of the receptor binding sites for certain ligands. However, at present, an explanation based on the limited reproducibility of ligand-binding studies in tissue samples is more likely.

We have further demonstrated that the recombinant CB2 activated G-proteins upon stimulation by specific agonists CP-55,940 and WIN 55,212-2 in an in vitro coupled assay. To our knowledge, this is the first such demonstration of functional activity for recombinant

cannabinoid receptor heterologously expressed in bacterial cells. It provides further firm support for the concept of using the *E. coli* expression host for production of functional GPCR for structural investigations. The activation of  $G_{\alpha i}$  was proportional to the quantities of the receptor-containing membranes. This indicated that this coupled assay may be used in further characterization of CB2 samples, either solubilized or reconstituted into a lipid matrix.

For the first time, our studies confirmed a ligand–CB2 receptor interaction in the micellar state by nuclear magnetic resonance spectroscopy. Our results unambiguously indicate that the solubilized receptor in DPC micelles is capable of binding the specific cannabinoid ligands CP-55,940 and anandamide with affinities similar to those reported for the membrane-localized receptor. The results confirmed that solubilization and purification procedures were at least partially successful. For the NMR experiments, the triple detergent mix was replaced by deuterated DPC using the Ni-NTA column. This could have reduced the amount of active protein. The successful ligand-binding studies on the fusion CB2 in the micellar state open up the possibility of studying structure and function of this receptor in solution.

We have developed approaches for reconstitution of the purified receptor into the lipid matrix, and followed success of the reconstitution by using fluorescent dye labeling of both lipid and protein. Ligand-binding studies (performed in both conventional filter-binding and Anopore-based format) have confirmed that at least a fraction of the reconstituted CB2 retained its functionality. It is our goal to enable structural studies on the recombinant protein under functional conditions. This is possible via formation of single, oriented lipid bilayers at the surface of a solid support containing functional membrane protein. By filling the pores of a porous substrate with single, tubular lipid bilayers we have been able to increase the accessible membrane surface area by more than two orders of magnitude compared to a flat solid surface. The subsequent gain in signal intensity enables structural studies by NMR and scattering techniques. Another advantage of the porous substrate is the fast and efficient removal of detergents used for GPCR solubilization and reconstitution, preventing losses of precious material.

In the ligand-binding assays, the use of porous substrates like Anopore greatly reduced nonspecific ligand binding. Furthermore, comparison of receptor activity suggested that function is not impaired by the presence of the solid support. We propose that this is the consequence of the existence of a water layer between the tubular lipid membrane and the pore surface as shown by us recently in NMR experiments (Gaede et al. 2004). At present, the concentration of the CB2-fusion in lipid bilayers was limited to about 5 wt% of membrane

material. This is significantly less than the 50 wt% of rhodopsin by weight in the rod outer segment disks (Stinson et al. 1991). While the current concentration is more than adequate for ligand-binding assays, structural studies on the protein may benefit from higher protein content. According to the amino acid sequence of the maltose-binding protein, this segment of the fusion has a strong negative charge at neutral pH. This imparts a strong negative surface potential on the membrane that is likely to limit incorporation of the CB2-fusion into lipid bilayers. We are currently working on strategies to cleave the CB2 fusion at the Tev-protease site without losing functional activity with the goal to further raise the concentration of functional protein in bilayers.

## Materials and methods

### Chemicals and reagents

[<sup>3</sup>H] CP-55,940 was purchased from Perkin-Elmer. The fluorescent dyes Texas Red (1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt), DiI C<sub>18</sub> (1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt), Alexa Fluor 532 carboxylic acid, and succinimidyl ester (<sup>1</sup>H-Pyrano[3,2-f:5,5-f']diindole-10,12-disulfonic acid, 5-[4-[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]phenyl-2,3,7,8-tetrahydro-2,3,3,7,7,8-hexamethyl) were purchased from Molecular Probes. Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs. The INDIA HisProbe-HRP kit for detection of poly-histidine-tagged fusion proteins was obtained from Pierce.

The cannabinoid ligands anandamide (*N*-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), CP-55,940 ((-)-*cis*-3[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol), ACEA (arachidonyl-2'-chloroethylamide/*N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), WIN 55,212-2 mesylate ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate), JWH015 ((2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone), AM281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide), AM630 (6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)-1*H*-indol-3-yl)(4-methoxyphenyl)methanone, (-)-cannabidiol (2-[(1*R*,6*R*)3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol), abn-CBD (4-[(1*R*,6*R*)3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol), and noladin ether (2-[(5Z,8Z,11 Z,14Z)-eicosatetraenyloxy]-1,3-propanediol) were purchased from Tocris. HU210 ((6*aR*)-*trans*-3-(1,1-dimethylheptyl)-6*a*,7,10,10*a*-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol), a specific central-type cannabinoid receptor synthetic agonist SR 141716A, and Δ<sup>9</sup>-tetrahydrocannabinol were kindly provided by Dr. G. Kunos (NIAAA, NIH).

The detergents 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), cholesteryl hemisuccinate Tris salt (CHS), and *n*-dodecyl-β-D-maltoside (DM) were obtained from Anatrace. *N*-Octyl-β-D-glucopyranoside (OG) was purchased from Calbiochem.

The lipids 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were obtained from Avanti Polar Lipids, Inc.

The deuterated chemicals D<sub>2</sub>O (99%), imidazole-d<sub>4</sub> (98%), and dodecylphosphocholine-d<sub>38</sub> were purchased from Cambridge Isotope Laboratories.

### Expression vectors, strains, and antisera

*E. coli* strain DH5α was obtained from Invitrogen. *E. coli* protease-deficient expression strain BL21 (DE3) was purchased from Stratagene. Anti-CB2 polyclonal antibody was obtained from Cayman Chemical. Anti-maltose-binding protein antibody was purchased from New England Biolabs. Plasmids encoding human CB2 gene (pRG611) and maltose-binding protein-thioredoxin-poly-histidine tag (pRG624 and pRG1023, derivatives of pRG/III-hs-MBP) were kindly provided by Dr. R. Grishammer (NIDDK, NIH) (Tucker and Grishammer 1996; Calandra et al. 1997; White et al. 2004). The plasmid for expression of hexa-histidine-tagged Tev protease (pRK793) was a gift from Dr. D.S. Waugh (NCI-Frederick, NIH) (Kapust et al. 2001).

### Construction of plasmids for CB2 expression

Plasmid pAY105 for expression of human CB2 protein (fused to the *E. coli* maltose-binding protein at the N-terminus, and to the *E. coli* thioredoxin followed by deca-histidine tag at the C-terminus) was constructed by ligating the 1.1-kb BamHI–NotI CB2-gene-encoding fragment of pRG611 into the 7.3-kb BamHI–NotI fragment of vector pRG624 (Fig. 1). The corresponding fusion protein CB2-105 consists of *E. coli* MBP (Lys1 to Thr391) followed by Gly-Ser, CB2 (Glu1 to Cys359), *E. coli* thioredoxin (TrxA, Ser2 to Ala109), Gly-Thr, and a deca-histidine tag (H10).

Plasmid pAY107 was constructed by ligating the 1.1-kb BamHI–NotI fragment of pRG 611 into the 7.5-kb BamHI–NotI fragment of vector pRG 1023. The resulting plasmid (Fig. 1) encodes a CB2-107 that contains two TEV protease-recognition sites flanking CB2 with residues Gly-Ser-Asn<sub>10</sub>-Glu-Asn-Leu-Tyr-Phe-Gln-Ser-Gly-Ser located upstream of CB2 and residues Ala<sub>3</sub>-Asn<sub>5</sub>-Gly<sub>3</sub>-Ser-Glu-Asn-Leu-Tyr-Phe-Gln-Ser-Gly<sub>3</sub>-Ser-Glu-Phe located downstream of CB2.

The expression vector is based on pMal-p2 plasmid (New England Biolabs), in which the *tac* promoter region of the plasmid was replaced with the *lac* promoter and the double ribosome-binding site of the vector pASK40 (Tucker and Grishammer 1996). Part of the M13 *ori* in this plasmid is replaced with the wild-type *hok/sok* gene cassette to prevent plasmid loss during prolonged fermentation (Tucker and Grishammer 1996).

### Expression of CB2 fusion protein in shake flasks

Expression of the recombinant receptor was carried out in *E. coli* cells DH5α and BL21(DE3). Cells were grown in double-strength YT-medium (Sambrook and Russell 2001) containing ampicillin (50 μg/mL) and 0.2% (w/v) glucose. For expression of functional CB2, *E. coli* cells containing the respective plasmids pAY105 or pAY107 were grown in 450 mL of medium in a 2-L flask at 37°C for 4–5 h until the optical density of the culture at 600 nm reached 0.4–0.5 units. Temperature was lowered to 20°C and synthesis of the recombinant protein induced by addition of 1 mM isopropyl-β-thiogalactoside (IPTG). Fermentation continued for another 38–40 h, and cells were collected by centrifugation, washed in phosphate-buffered saline, and stored at –80°C.

### Membrane preparation

Spheroplasts of *E. coli* and a cytoplasmic membrane fraction were prepared according to the protocols by Weiss (1976) and Tai and Kaplan (1985) with the following modifications: *E. coli* cells were collected by centrifugation, washed twice with 0.1 M Tris-HCl (pH 8.0) buffer, and resuspended in 0.1 M Tris-HCl (pH 8.0) buffer containing 20% (w/v) sucrose, to adjust an optical density of the cell suspension to 10 at 600 nm. A cocktail of protease inhibitors (Roche) was added at this stage. The temperature was adjusted to 37°C, and a solution of lysozyme (2 mg/mL) was added slowly, under constant stirring, until the final concentration of lysozyme of 0.1 mg/mL was reached. The cell suspension was incubated at 37°C for another 15 min with stirring. A solution of 0.1 M EDTA (pH 7.0) was added slowly under continuous stirring until the final concentration of EDTA of 10 mM was reached. The incubation continued for another 10 min.

Spheroplasts were centrifuged at 12,000g for 20 min. The pellet was collected and washed once with 0.1 M Tris-HCl (pH 8.0) containing 20% sucrose. Spheroplasts were centrifuged again at 12,000g for 20 min. The pellet of spheroplasts was resuspended in ice-cold water, which resulted in osmotic lysis. The remaining intact cells were broken up by mild sonication. Immediately, 1 M Tris buffer (pH 8.0) containing protease inhibitors was added to produce a final Tris concentration of 50 mM. A 1 M solution of MgCl<sub>2</sub> was added to achieve a final concentration of 1 mM. A solution of DNase I was added, the cell-free extract was briefly sonicated, and further incubated on ice for 1 h. The extract was centrifuged at 150,000g for 1 h, and the membrane pellet was washed with Tris buffer and centrifuged again at 150,000g for 1 h. The membrane pellet was resuspended in a small volume of Tris buffer, frozen in liquid nitrogen, and stored at -80°C.

### Solubilization of CB2 fusion protein

Solubilization was carried out at 4°C. Frozen cells (80 g) were homogenized in 600 mL of solubilization buffer (50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 0.5% [w/v] CHAPS, 0.1% [w/v] CHS, 1% [w/v] DM, 5 mM MgCl<sub>2</sub>, and 30% glycerol) supplemented with DNase I (10 µg/mL) and EDTA-free protease-inhibitor cocktail (Roche). The mixture was sonicated for 15 min (Branson Sonifier 250, 1/2-inch flat tip, output 6, duty cycle 50%). After stirring the extract for another 40 min, the cell debris was removed by centrifugation (Beckman 70Ti rotor, 50,000 rpm, 1 h). The sample was passed through a 0.2-µm filter and purified by immobilized metal affinity chromatography.

### Purification of the CB2-107 fusion protein

Purification of the CB2-107 fusion protein was performed with a 20-mL Ni-NTA agarose (QIAGEN) column at a flow rate of 0.5 mL/min in buffer A (50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 30% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% DM), on an AKTA-Purifier 100 system equipped with a sample valve S1 with air sensor and a fraction collector Frac950. After loading of the protein extract, the column was washed with 10 column volumes of 30 mM imidazole in buffer A and recombinant protein eluted with a linear gradient of imidazole in buffer A. Fractions of 4 mL were collected and analyzed for the presence of CB2 fusion protein by Western blot. Fusion CB2 protein

was eluted at imidazole concentrations in the range from 80 to 150 mM.

Fractions containing recombinant CB2 were pooled together and concentrated 10-fold on an Apollo 20-mL centrifugal spin concentrator (hydrophilic membrane, 70 kDa molecular mass cutoff; Orbital Biosciences). Concentrated fractions were diluted 10-fold with buffer B (50 mM Tris-HCl at pH 7.5 containing 30% glycerol, 0.5% CHAPS, 0.1% CHS, and 0.1% DM) and loaded onto a Source 30Q column (7.6-mL volume; Amersham). Recombinant CB2 was eluted with a linear gradient of NaCl in buffer B. Fractions (4 mL) were collected and analyzed for the presence of recombinant CB2 by Western blot. Fractions containing CB2 were pooled together and concentrated using a centrifugation filter device.

### Preparation of CB2 labeled with fluorescent dye Alexa Fluor 532

Recombinant CB2 protein (1 mg) in buffer A, purified by chromatography on Ni-NTA- and Source 30Q columns as described above, was incubated with 2 mL of a Ni-NTA agarose slurry (QIAGEN) overnight on a shaker. Resin retaining immobilized protein was packed into a 10-mL EconoPack column, and the buffer was exchanged by passing 30 column volumes of 50 mM sodium phosphate (pH 7.5) containing 30% glycerol, 0.5% CHAPS, and 0.1% DM. The resin was resuspended in 2 mL of sodium phosphate buffer with detergents, and 100 µL of Alexa Fluor 532 reactive dye (succinimidyl ester, Molecular Probes) in 0.1 M Na-bicarbonate buffer was added. After incubation for 2 h on a shaker, the resin was transferred into a small EconoPack column and washed with 50 column volumes of buffer A to remove the nonreacted dye. Labeled protein was eluted with 200 mM imidazole in buffer A and concentrated in a centrifugal spin concentrator (Orbital Bioscience). All steps were performed at 4°C. Efficiency of labeling was determined by measuring absorbance of the conjugate solution at 280 nm and 530 nm, according to the protocol recommended by the manufacturer (Molecular Probes).

### Preparation of purified recombinant CB2 for solution NMR experiments

The purified preparation of CB2-107 (6 mg) was mixed with 5 mL of Ni-NTA agarose (QIAGEN) in buffer A and incubated overnight at 4°C. Resin with retained CB2 was packed into the EconoPack column (BioRad) and washed with 10 column volumes of buffer A, followed by 10 column volumes of buffer C (10 mM sodium phosphate buffer at pH 7.5 containing 150 mM NaCl and 15 mM deuterated [d<sub>38</sub>, 98%] DPC in D<sub>2</sub>O). Recombinant CB2 was eluted with 4 column volumes of 200 mM deuterated imidazole (d<sub>4</sub>, 98%) in buffer C and concentrated 10-fold using a concentrating filter device (Orbital Biosciences).

### Processing of CB2-107 fusion protein by the Tev protease

#### Expression and purification of Tev protease

Expression of the Tev protease mutant S219V as a fusion with MBP, and in vivo autoproteolysis was performed using strain BL21-RIL/ pRK793, as described in Kapust et al.

(2001). Cells were disrupted by sonication and the hexa-histidine-tagged Tev protease purified by chromatography on Ni-NTA agarose as described in White et al. (2004). Fractions containing recombinant Tev were pooled together, concentrated on a Centricon centrifugal concentrating device (Millipore), mixed 1:1 with glycerol, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . The concentration of purified Tev protease was 5.6 mg/mL.

#### *Cleavage of CB2-107 fusion protein by Tev protease*

To determine the amount of His-Tev (S219V) protease necessary to perform proteolytic cleavage at the two Tev-recognition sites located on the CB2-107 fusion protein, purified recombinant receptor (concentrated eluate from Ni-NTA column in buffer B) was incubated at  $4^{\circ}\text{C}$  with variable concentrations of Tev protease in the presence of 1 mM dithiothreitol. Cleavage of the fusion protein was monitored by analyzing the reaction products by SDS-PAGE and a Western blot. Complete cleavage of CB2-107 was achieved at a ratio of 1:0.5 (CB2: Tev, M/M) after 16 h of incubation.

#### *Protein analysis*

Recombinant CB2 fusion protein synthesized in *E. coli* was detected by Western blot with anti-MBP sera (1:2000; New England Biolabs), anti-CB2 polyclonal antibody (1:2000; Cayman Chemical), or with anti-His INDIA-HRP reagent (Pierce). The Western blot was developed with anti-rabbit IgG conjugated with fluorescein (Amersham) and scanned on a Typhoon 8600 fluorescent scanner (Amersham).

Protein concentration in the presence of high detergent concentrations was determined using a Dc protein assay reagent (BioRad).

#### *CB2 binding assays*

The ligand-binding analysis with [ $^3\text{H}$ ] CP-55,940 (Perkin-Elmer) on *E. coli* membranes was performed essentially as described in Felder et al. (1992) using a Brandel cell-harvesting device. Briefly, *E. coli* membranes were incubated with 0.5 nM [ $^3\text{H}$ ] CP-55,940 and different concentrations of unlabeled ligands for 60 min at  $30^{\circ}\text{C}$ . The incubation (binding) buffer consisted of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 3 mM  $\text{MgCl}_2$ , and 0.14 % (w/v) bovine serum albumin. Nonspecific binding was determined in the presence of 1  $\mu\text{M}$  unlabeled CP-55,940. Free and bound radioligands were separated by rapid filtration through pre-soaked Whatman GF/B filters. The filters were washed three times with 4 mL of cold binding buffer, and the amount of radioisotope retained on the filter was determined by liquid scintillation counting. Data from ligand binding were analyzed using GraphPad Prism Software.  $\text{IC}_{50}$  and  $K_i$  values were determined through nonlinear regression analysis.  $K_d$  and  $B_{\text{max}}$  values were determined from saturation binding experiments.

#### *Ligand-binding assay on Anopore filters*

Anopore filters (Whatman) were used as an alternative method to perform the ligand-binding assay on the *E. coli* membranes expressing recombinant cannabinoid receptor. Briefly, the pro-

cedure used to perform the binding assay was as follows. Anopore filters (25 mm diameter, 0.2  $\mu\text{m}$  pore size) were pre-loaded with the *E. coli* BL21-107 membranes expressing CB2 by slowly passing a membrane suspension in 50 mM Tris-HCl (pH 7.5) buffer through the filter on a Millipore vacuum-filtration manifold. Filters were washed by passing 10 mL of binding buffer containing 50 mM Tris (pH 7.5) buffer, 3 mM  $\text{MgCl}_2$ , and 0.1% bovine serum albumin. Then 1 mL of the solution of radiolabeled ligand and variable concentrations (from  $1 \times 10^{-11}$  M to  $1 \times 10^{-5}$  M) of unlabeled ligand (CP-55,940) in binding buffer was applied to the Anopore filter and slowly passed through the filter after vacuum was applied. Filters were briefly washed three times with 2 mL of binding buffer and immersed in the scintillation liquid, and activity was counted.

#### *Reconstitution of CB2 receptor with G-protein subunits*

The receptor-catalyzed  $\text{GDP} \rightarrow [\text{S}^35]\text{GTP}\gamma\text{S}$  exchange was measured by incubation of variable amounts of urea-treated membrane preparations of CB2-107 with the  $\text{G}_{\alpha i}$  subunit in the presence of a saturating concentration (1  $\mu\text{M}$ ) of  $\text{G}_{\beta\gamma}$  subunits, essentially as described in Glass and Northup (1999). Urea-treated membranes were prepared as follows. The BL21-CB2 membrane preparation (3 mL of a 20 mg/mL protein concentration) was diluted with 11 mL of 10 mM MOPS (pH 7.5) buffer on ice. After centrifugation at 35,000 rpm for 20 min in a 45 Ti rotor, the supernatant was discarded and the pellet was resuspended in 8 mL of 7 M urea in 10 mM MOPS (pH 7.5) buffer. After incubation on ice for 30 min, the suspension was diluted with 6 mL of 10 mM MOPS buffer, and the membranes were precipitated by centrifugation at 50,000 rpm for 30 min. The pellet was resuspended in 10 mM MOPS buffer and centrifuged at 35,000 rpm for 20 min. The resulting pellet was resuspended on ice in 2 mL of 10 mM MOPS buffer containing 12% sucrose, and stored at  $-80^{\circ}\text{C}$ .

For the G-protein-coupled assay, *E. coli* CB2-107 membrane preparations were resuspended in the reaction buffer (50 mM MOPS buffer at pH 7.5 containing 1 mM EDTA, 1 mM  $\text{MgSO}_4$ , 1 mM DTT, 1 mg/mL bovine serum albumin), and G-protein-binding activity was measured upon activation of recombinant receptor with agonists CP-55,940 or WIN 55,212-2 mesylate (concentration 1  $\mu\text{M}$ ). A 30-min incubation was started by supplementing the reaction mixture with 200 nM  $\text{G}_{\alpha i}$  and saturating concentrations (1  $\mu\text{M}$ ) of  $\text{G}_{\beta\gamma}$  and terminated by rapid addition of 2 mL of cold wash buffer (20 mM Tris-HCl at pH 8.0, 100 mM NaCl). The reaction mixture was filtered over nitrocellulose membrane on a Millipore vacuum manifold. Experiments were performed in siliconized test tubes to decrease nonspecific binding of the receptor to the tube surface.

#### *CB2-107 ligand-binding studies in the micellar state by NMR*

Proton high-resolution NMR experiments were conducted at a resonance frequency of 500.13 MHz on a DMX500 NMR spectrometer (Bruker Biospin) equipped with an inverse triple resonance probe head with actively shielded x,y,z gradient coils. The signal of protonated water was suppressed by a Double Pulsed Field Gradient Spin Echo as described in Hwang and Shaka (1995). Depending on ligand concentration, from 500 to 10,000 scans with a delay time of 6 sec were acquired. Sample

temperature was controlled to  $4.0 \pm 0.1^\circ\text{C}$  using a Bruker BVT2000 variable temperature accessory. Data were analyzed using XWINNMR 3.1 software (Bruker, Biospin).

Experiments were conducted on 600  $\mu\text{L}$  of a CB2 solution in DPC micelles. The ligands CP-55,940 and anandamide were added as stock solution in DPC. The ligand stock solutions contained equimolar amounts of DMSO to provide a standard for calibration of ligand resonance intensity. The ligands were added directly into the NMR tube. Samples were homogenized after every addition of ligand by inverting the sealed tubes repeatedly with a motorized device for 15 min at ambient temperature.

#### *Reconstitution of CB2-107 into lipid bilayers and deposition into Anopore membranes*

The solution of CB2 in 50 mM Tris-HCl buffer containing a mixture of the triple detergents (0.1% DM/0.1% CHAPS/0.1% CHS), 100 mM NaCl, and 30% glycerol was mixed with an appropriate amount of a 20 mg/mL solution of SOPC in 3% OG to achieve a ratio of protein to lipid of 1:1 (w/w). For fluorescence studies, 1  $\mu\text{g}$  of fluorescently labeled lipid (Dil C18, 1 mg/mL solution in methanol) was added per 1 mg of SOPC as well as 1  $\mu\text{g}$  of CB2 labeled with fluorescent dye Alexa Fluor 532 (efficiency of labeling: 3.6 molecules of dye per molecule of CB2) added per 1 mg of unlabeled purified CB2. The CB2 and SOPC mixture was rapidly diluted 20-fold into 50 mM Tris-HCl buffer, incubated at room temperature for 10 min, and passed (twice) through a wet Anopore filter (0.2  $\mu\text{m}$  pore size, 25 mm diameter). Filters were washed with Tris-HCl buffer and scanned on a Typhoon 8600 fluorescence scanner (Amersham Biosciences). DilC<sub>18</sub> was excited at 633 nm, and emission was detected at 670 nm (photomultiplier setting 500, normal sensitivity). Alexa Fluor 532 was excited at 532 nm and emission was registered at 610 nm (PMT setting 500, normal sensitivity). Quantitation of the amounts of lipid and protein deposited into the Anopore filter was performed using the computer program ImageQuant.

#### *Confocal microscopy on proteoliposomes*

Two-photon images were obtained on a Zeiss 510 META/NLO scan head attached to an upright Zeiss Axioplan 2 microscope. A modelocked Ti:sapphire laser (Coherent Chameleon) tuned to 810 nm was used for simultaneous multiphoton fluorescence excitation of Alexa Fluor 532 and Texas Red. Dual labeled CB2-containing liposomes were imaged using a 20 $\times$  NA 0.5 water objective. All 32 channels of the META photomultiplier array (spanning 376–719 nm) were used to collect the complex fluorescent spectrum emitted from the labeled CB2 and lipid. The isolated signals from Alexa 532 and Texas Red were deconvoluted from spectral images by linear unmixing using reference spectra of the pure labels.

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#### Conflict of interest

The reconstitution procedure described in this manuscript using anodic aluminum oxide porous filters is one of the examples of a pending U.S. patent application DHHS reference number E-011-2004/2-PCT-1 submitted on behalf of the U.S. Department of Health and Human Services. The authors do not have any other competing interests.

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